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INFLUENCE OF OZONE ON XENOBIOTIC METABOLISM

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INTRODUCTION

Gardner et al. (1) found that ozone (O_3) exposure increased pentobarbital (PEN)-induced sleeping time (S.T.) in female CD-1 mice. The animals were exposed to 1960 μg O_3/m^3 (1 ppm) for 3 hr/day for up to 7 days, but an effect was only observed on the second and third day of exposure. Since animals awake from PEN-induced S.T. because of hepatic xenobiotic metabolism (2-5), it was hypothesized that O_3 exposure resulted in effects that occurred in the liver, making investigations to elucidate the mechanisms involved of interest. To this end, a series of studies were performed which will be summarized here. The reader is referred to papers (6-9) for a complete description of the investigations.

METHODS

Materials and methods have been described in detail (6-9), but they will be briefly recounted here.

Animals were caged individually during exposure to air or O_3 within a Rochester-type chamber. O_3 was generated from 100% O_2 with a commercial ozonizer and was monitored continuously with a chemiluminescence O_3 analyzer which was calibrated every two weeks using the Federal Register technique. On a given experimental day, one group of animals was exposed to O_3 and another was exposed under similar conditions to clean room air which had been filtered through activated charcoal and HEPA filters.

All drug-induced S.T. studies were begun routinely at 1:00 pm ± 15 min and were conducted in an isolated temperature and humidity controlled room. All drug injections were IM. The following doses were used for S.T. studies: 50 mg/kg sodium pentobarbital (Nembutal, PEN) (mice), 25

mg/kg PEN (rats), 40 mg/kg PEN (hamsters), 60 mg/kg hexobarbital (mice), 6.25 mg/kg sodium thiopental (mice) and 60 mg/kg zoxazolamine (mice). The time to loss of righting reflex (i.e. the time between injection and the animal remaining on its back) and S.T. (the time between the loss and regaining of the righting reflex) were measured with a stopwatch. Because the time to the loss of righting reflex was rarely altered by any treatment, it will not be discussed further. In the case of zoxazolamine, paralysis time was measured equivalently to that of S.T., except that leg paralysis was the indicator.

Both inducers and inhibitors of mixed function oxidases were used. The inducers [phenobarbital, pregnenolone-16α-carbonitrile (PCN), β-naphthoflavone (BNF), and PEN] were administered for varying days (depending on the inducer) prior to O_3 exposure in dosages used by Haugen et al. (10), except for PEN for which other doses (7) were used. The competitive inhibitors (SKF525A and piperonyl butoxide) were injected immediately before O_3 exposure. In preliminary experiments, it was determined that the specific vehicles used caused no significant effect on PEN-induced S.T. compared to saline or non-injected controls (7).

Hepatic mixed function oxidases and cytochrome P-450 levels were measured as described (8) using slight modifications of conventional methods.

Pharmacokinetic studies of ^{14}C-PEN and measurement of metabolites were conducted as described (9) using high-pressure liquid chromatography, fraction collection, and liquid scintillation counting. Pharmacokinetics of non-radiolabelled PEN were conducted using similar liquid chromatography techniques.

The basic statistical method used for S.T. and enzyme activity involved analysis of variance followed by selected multiple comparison procedures to elucidate patterns of significant differences. Each daily replication of a given design included all factors that were to be compared in the experiment. The minimum number of animals per treatment group was 6, and the maximum number was based upon logistical restrictions. Each experiment consisted of at least 2 replicates, except for the experiments with radiolabelled PEN.

For PEN pharmacokinetic studies (9), nonlinear estimation techniques were used to fit the curves, and least squares estimates of the parameters were obtained using the iterative Marquardt (11) method.

RESULTS

Sex, strain and species sensitivity. To evaluate female mouse strain sensitivity, one outbred (CD-1) and two inbred (C57BL/6N and DBA/2N) strains were compared (6). O_3 (1960 μg/m³, 1 ppm x 5 hr) increased PEN-induced S.T. ($p = 0.0001$) in all strains. To determine whether this effect were sex or species specific, male and female CD-1 mice, rats, and hamsters were exposed for 5 hr to 1960 μg O_3/m³ (1 ppm) (6). The females of all species exhibited an increased PEN-induced S.T. ($p < 0.05$). Male mice and rats were not affected ($p > 0.1$). Male hamsters had an increase in S.T. ($p < 0.1$), but less ($p = 0.075$) than that of the females.

Influence of concentration and number of exposures. Female CD-1 mice were exposed to 196-9800 μg O_3/m³ (0.1-5 ppm) for 3 hr/day for a varying number of days (Table 1) (6). As the concentration of O_3 was

TABLE 1

EFFECT OF O_3 ON PENTOBARBITAL-INDUCED SLEEPING TIME IN FEMALE CD-1 MICE[a]

O_3 Concentration (ppm)	Days of Exposure (3 hr/day)	n	Sleeping Time, min		
			Air	O_3	O_3-Air
5.00	1	46-50	22.6 ± 0.8[b]	57.9 ± 2.4[c]	35.3
1.00	1	73-74	22.8 ± 0.7	31.0 ± 1.3[c]	8.2
	2	62-83	23.0 ± 0.8	33.5 ± 1.0[c]	10.5
	3	44	19.9 ± 0.5	24.2 ± 1.0[c]	4.3
0.50	1	15	26.9 ± 1.2	27.0 ± 1.3	0.1
	2	15	22.9 ± 0.4	26.4 ± 0.3[c]	3.5
	3	15	28.4 ± 1.0	44.8 ± 1.5[c]	16.4
	4	15	19.5 ± 0.3	21.0 ± 0.8	1.5
0.25	1	15	18.8 ± 0.3	19.1 ± 0.2	0.3
	2	15	21.2 ± 0.4	22.5 ± 0.2	1.3
	3	15	20.6 ± 0.1	21.6 ± 0.2	1.0
	4	15	19.4 ± 0.2	20.5 ± 0.3	1.1
	5	30	21.5 ± 0.3	21.7 ± 0.4	0.2
	6	30	20.8 ± 0.3	32.5 ± 0.9[c]	11.7
	7	30	22.1 ± 0.4	24.9 ± 0.7[c]	2.8

0.10	14	41-42	26.3 ± 1.2	25.1 ± 1.3	-1.2
	15	40-44	26.9 ± 1.3	30.1 ± 1.5[c]	3.2
	16	41-42	22.7 ± 1.3	26.5 ± 1.8[c]	3.8
	17	41	26.3 ± 1.3	26.8 ± 1.5	0.5

[a]Data from reference 6

[b]Mean ± S.E.

[c]Air and corresponding O_3 mean are different ($p < 0.05$)

decreased, increasing numbers of daily 3 hr exposures were required to significantly increase PEN-induced S.T. Once the maximal effect occurred, increasing the number of exposures resulted in a diminution of the effect. Generally, effects were observed around an approximate C X T (concentration, ppm x time, hr) of 5. Also, CD-1 female mice were exposed to four different concentrations of O_3 (1960 to 196 $\mu g/m^3$, 1 - 0.1 ppm) for 5-20 hr continuously in a fashion yielding a C x T of 5 (6). All regimens increased ($p < 0.01$) PEN induced S.T. The time to recovery was examined in mice exposed to 1960 μg O_3/m^3 (1 ppm) for 5 hr/day (6). Recovery was complete within 24 hr after exposure, whether exposure was for 1, 2, or 3 days.

Effect on S.T. or paralysis time with various drugs. To determine whether the previous responses were specific to PEN, other drugs having known and different mechanisms for termination of action were used in CD-1 female mice exposed to 1960 μg O_3/m^3 (1 ppm) for 5 hr (7). O_3 increased ($p < 0.05$) S.T. induced by hexobarbital and thiopental and paralysis time induced by zoxazolamine.

Influence of mixed function oxidase inducers on PEN-induced S.T. CD-1 female mice pretreated with mixed function oxidase inducers with partially characterized mechanisms of action were exposed to 1960 μg O_3/m^3 (1 ppm) or air for 5 hr prior to measurement of PEN-induced S.T. (7). Pretreatment with PEN caused no differences ($p > 0.1$). However, pretreatment with BNF, PCN, and phenobarbital caused a decrease ($p \leq 0.006$) in PEN-induced S.T. In all groups, O_3-exposed animals exhibited a longer ($p \leq 0.001$) PEN-induced S.T. than air controls.

Because BNF is an inducer in C57BL but not DBA mice, an additional experiment was performed with these strains and compared to a similar study with CD-1 mice (7). Preliminary studies had shown that CD-1 mice had an apparent phenotype similar to that of the C57BL mice with respect to BNF induction of mixed function oxidases. DBA mice responded similarly to CD-1 mice as described above. However, in C57BL mice, although BNF reduced ($p = 0.006$) PEN-induced S.T., O_3 had no significant effect in the BNF-pretreated mice.

Effect of competitive inhibitors of mixed function oxidases on PEN-induced S.T. SKF525A and piperonyl butoxide increased PEN-induced S.T. in CD-1 female mice with increasing doses (7). There was also a trend towards an increased magnitude of the O_3 (1960 μg/m³, 1 ppm x 5 hr) effect as the concentrations of inhibitors were increased.

Effect on hepatic microsomal enzymes in CD-1 female mice. A 3 hr exposure to concentrations of O_3 as high as 9800 μg O_3/m^3 (5 ppm) did not change the concentration of cytochrome P-450 or the activities of aminopyrine N-demethylase or p-nitroanisole O-demethylase (8). However, this exposure regimen and another (1960 μg O_3/m^3, 1 ppm, x 5 hr/day x 2 days) increased slightly ($p < 0.05$) the activity of aniline hydroxylase.

Effect on pentobarbital pharmacokinetics in female CD-1 mice. A 3 hr exposure to 9800 μg O_3/m^3 (5 ppm) did not affect brain concentrations of PEN at time of awakening, even though S.T. was increased (9). A similar exposure regimen also did not alter the pattern of brain or plasma metabolites at various times up to 90 min. post-exposure (9). Following this exposure, first-order clearance kinetics of PEN were observed in both the air and O_3 groups, and O_3 increased the plasma $t_{1/2}$ by 106% (9). Mice exposed to 1960 μg O_3/m^3 (1 ppm) for 5 hr had a 71% increase in the plasma half-life of PEN using non-radiolabeled PEN (unpublished data). For the air group, the exponential model was $y = 53.81e^{-0.0289t}$, and the $t_{1/2}$ was 24.0 min. For the O_3 group, the exponential model was $41.4e^{-0.0169t}$, and the $t_{1/2}$ was 41.0 min. Clearance followed first-order kinetics with a one-compartment model in this experiment also (unpublished data).

DISCUSSION

It appears that the effect of O_3 on PEN-induced S.T. is not specific to strain of mouse or species of animal, but it is sex specific, with females being more sensitive. For most species (mice, hamsters, monkeys, man, etc.) except rats, there is only a very small sex difference in xenobiotic metabolism (12). The reason for the sex difference in the effect of O_3 is unknown.

In addition, the response is not specific to PEN. O_3 also altered the mechanisms of the termination of action of hexobarbital, zoxazolamine, and thiopental. Within the liver, PEN and hexobarbital metabolism are more related to cytochrome P-450 than to cytochrome P-448-dependent

activities (12-18). Zoxazolamine metabolism is more related to cytochrome P-448 than to cytochrome P-450 (17-20). Other major differences in the nature of the metabolism (i.e. aliphatic vs. aromatic) also exist between these 3 drugs (2, 19-22). In contrast to the above, sedation from thiopental is terminated because of drug redistribution (23,24). Thus, it would appear that O_3 might affect some aspects of both drug redistribution and metabolism. Because of the different mechanisms involved in hexobarbital, zoxazolamine, and PEN metabolism, it is also possible that some common component(s) of metabolism may have been altered.

There is no direct evidence to explain the pattern of tachyphylaxis observed in the concentration-response studies. Nor can the potential mechanism responsible for the findings that as concentration decreases, it takes an increasing number of intermittent daily exposures to affect PEN-induced S.T. be explained easily. Since an approximate CxT of 5 produces the maximal effect at the target site(s), it is possible that as exposure continues, damage increases, reaching a point where an undefined feedback repair mechanism occurs. At a CxT > 5, this hypothetical repair process might begin to overcome the damage process, resulting in a diminution of the magnitude of the O_3 effect. This proposed mechanism is supported by the equivalency of the maximal magnitudes of effects for various concentrations of O_3 ($\leq$ 1960 $\mu g/m^3$, 1 ppm).

In the studies of CD-1 mice pretreated with PCN, BNF, and phenobarbital, similar observations were made in that O_3 increased PEN-induced S.T. to an equivalent extent in both inducer- and vehicle-pretreated animals. This occurred even though these agents differentially induce 4 isozyme forms of the hepatic cytochrome P-450 system (10). It may be assumed that it is unlikely that phenobarbital, BNF and PCN induced PEN

metabolic systems to an equivalent extent. If this assumption were correct, then differing levels and types of induction did not alter the magnitude of the O_3 effect. Thus, again there is a suggestion that some common pathway(s) of drug metabolism is being altered.

To evaluate directly the effects of O_3 on hepatic mixed function oxidases, enzyme activities and the level of cytochrome P-450 were determined in vitro. Except for a slight increase in aniline hydroxylase, no changes were found. Goldstein et al. (25) also found no effect of O_3 on liver cytochrome P-450 levels. Hepatic benzo(a)pyrene hydroxylase activity was unchanged by O_3 exposure (26).

Because of the lack of effects with these in vitro methods of studying metabolism, in vivo methods were applied. O_3 did not qualitatively affect metabolite patterns, however metabolism was quantitatively reduced. The potential for an effect of O_3 exposure on PEN receptors in the brain was assessed by measuring the brain concentrations of PEN at time of awakening. No differences were found, so apparently O_3 does not affect the sensitivity of the brain to PEN.

In summary, the mechanism(s) for the effect of O_3 on PEN-induced S.T. are not known definitively. However, it is hypothesized that some common aspect(s) of drug metabolism is quantitatively reduced. In addition, drug redistribution is apparently slowed. It is unlikely that O_3 itself caused these effects at target sites distant from the lung (27). Because of the free radical nature of oxidation initiated by O_3, a myriad of oxygenated products, several of which have toxic potential, may be formed in the lung (28-30). However, the stability of such products in the blood and their reaction with organs such as the liver are speculative at present.

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